

The role of flavonoids in the establishment of plant roots endosymbioses with arbuscular mycorrhiza fungi, rhizobia and *Frankia* bacteria

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Key words: flavonoids, endosymbiosis, legume-rhizobium symbioses, actinorhizal symbioses, arbuscular mycorrhiza, signaling

Flavonoids are a group of secondary metabolites derived from the phenylpropanoid pathway. They are ubiquitous in the plant kingdom and have many diverse functions including key roles at different levels of root endosymbioses. While there is a lot of information on the role of particular flavonoids in the Rhizobium-legume symbiosis, yet their exact role during the establishment of arbuscular mycorrhiza and actinorhizal symbioses still remains unclear. Within the context of the latest data suggesting a common symbiotic signaling pathway for both plant-fungal and plant bacterial endosymbioses between legumes and actinorhiza-forming fagales, this mini-review highlights some of the recent studies on the three major types of root endosymbioses. Implication of the molecular knowledge of endosymbioses signaling and genetic manipulation of flavonoid biosynthetic pathway on the development of strategies for the transfer and optimization of nodulation are also discussed.

Introduction

Flavonoids are secondary metabolites derived from the phenylpropanoid pathway and >9,000 flavonoids have been characterized in plants.¹ Major subgroups of flavonoids that are found in most higher plants include chalcones, flavones, flavonols, anthocyanins, proanthocyanidins (condensed tannins) and aurones.^{2,3} Diverse functions of flavonoids include UV protection, sexual reproduction process, defense and flower coloring. Even if complex, the biosynthesis of these molecules has been well described and numerous enzymatic steps are involved (Fig. 1).^{4,5} In *Arabidopsis* most enzymes implicated in flavonoid biosynthesis are encoded by single copy genes, whereas in most plant species most genes occur in multigene families.⁶ Flavonoids are synthesized in the cytosol,⁷ stored in vacuoles,⁸ and they are also known to be present in external rhizosphere through exudation.⁹ Moreover, flavonoid synthesis is organ and tissue-dependent, and is affected by environmental conditions, such as light intensity, temperature and nitrogen.¹⁰⁻¹² Flavonoids accumulate specifically in dividing cells in the root, for example in the lateral root

and nodule primordia of subterranean clover (*Trifolium subterraneum*),^{13,14} in the root tip and the lateral root primordia of *Arabidopsis*.^{15,16}

Plant roots form three major types of intracellular endosymbioses in symbiotic relationships with arbuscular mycorrhizal (AM) fungi of the group of Glomeromycetota and with nitrogen fixing bacteria of the genus *Rhizobium* and *Frankia*. At least 80% of all angiosperms are able to participate in AM symbiosis while only ten families of angiosperms are known to form symbiotic association with nitrogen-fixing bacteria in root nodules.¹⁷ In addition to occurring with rhizobia in the legumes and Ulmaceae, nitrogen-fixing symbioses involving root nodules also occur with *Frankia* in some members of *Betulaceae*, *Casuarinaceae*, *Coriariaceae*, *Datisceae*, *Elaeagnaceae*, *Myricaceae*, *Rhamnaceae* and *Rosaceae*. Molecular phylogeny of plant groups that engage in root nodule symbiosis shows that they all belong to a single clade, the Fabid (Eurosoid 1).¹⁸ Some features of root nodule endosymbiosis could have been recruited from the more ancient AM symbiosis.^{19,20} Hence, the question of shared mechanisms including the control of early signaling events is raised.^{21,22} Orchestration of these early events requires the exchange of signaling molecules. The rhizobial signal molecule, the so-called nodulation (Nod) factors are lipochitooligosaccharides (LCOs), consisting of an N-acetylglucosamine backbone, N-acylated on the terminal non-reducing sugar and bearing different substitutions on the oligosaccharidic backbone that is symbiosis specific.²³ In the case of actinorhizal symbiosis, *Frankia* signals are unknown.^{24,25} However, it has been previously shown that *Frankia alni* (ACN14a) produces a root hair deforming factor (RHDF) in culture supernatant that reacts with *Alnus glutinosa* root hair cells inducing branching and curling of root hair cells.²⁶ This factor was shown to have a molecular weight below 3,000 da, to be heat-stable (similar to Rhizobium Nod factor) but also to be hydrophilic and to resist to chitinases (contrary to Rhizobium Nod factor). In AM symbiosis, it has been suggested that LCOs produced by the AM fungus could have a role in the establishment of AM symbiosis.²⁷ The work on the diversity and the role in discriminating specificity of LCOs produced by AM fungus is still in progress.²⁷ The recent availability of genomic resources in AM fungi is opening new possibilities to characterize the genes involved in the synthesis of signaling molecules.²⁸

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Submitted: 03/13/12; Accepted: 03/15/12
<http://dx.doi.org/10.4161/psb.20039>

which is the site for *Rhizobium* infection.⁵¹ Previous studies with *Sinorhizobium meliloti* revealed that certain flavonoids serve as chemo-attractants for rhizobia in a specific manner to promote bacterial movement toward the roots for establishing contact, colonization and infection leading to nodule development.⁵²⁻⁵⁵ It has also been demonstrated that plant flavonoids act as growth regulators of rhizobia.⁵⁵ For example, daidzein, luteolin-7-O-glucoside and quercetin-3-O-galactoside from alfalfa were found to enhance the growth of *Bradyrhizobium japonicum*,⁵⁶ and *S. meliloti*,⁵⁷ respectively.

Flavonoids regulate *nod* genes expression. In legumes specific flavonoids released from the roots interact with the Nod D protein of *Rhizobium* to activate transcription of other *nod* genes responsible for the synthesis of lipochitooligosaccharides called Nod factors.²³ This combination of Nod D proteins with flavonoids triggers the production of highly specific Nod signals which trigger different modifications in plants including, curling the tip of a root hair back on itself, trapping the bacteria within a pocket, from which they are taken up into a plant-made intracellular infection thread.⁵⁸ In addition, the Nod factors also induce cell division, gene expression in the root cortex and pericycle for starting the development of the nodule.^{59,60} Numbers of flavonoids have been shown to induce transcription of nodulation genes.⁶¹ This host specificity of rhizobia results in part from a Nod D-dependent upregulation of *nod* genes in response to a cocktail of flavonoids in the host plant's root exudates. In *Sinorhizobium meliloti* it was shown that whereas several flavonoids stimulated the DNA binding affinity of Nod D1 to *nod* gene promoters only luteolin is capable of promoting the downstream changes necessary for *nod* gene induction.⁶²

Flavonoids and nodule development. The plant hormone auxin (3-indolyl acetic acid, IAA) controls virtually all plant developmental and physiological processes including stimulation of the first divisions in pericycle,⁶³ which lead to lateral root development.⁶⁴ Auxin is synthesized locally in shoot apices, leaf primordial and developing seeds, and is then transported to the subapical tissues by polar auxin transport.⁶⁵ It has been suggested that Nod factor perception could induce certain flavonoids that inhibit auxin transport causing local auxin accumulation at the nodule initiation site leading to the initiation of nodule primordia.^{66,67} This was recently demonstrated by silencing of chalcone synthase in *M. truncatula* roots using RNA interference.⁶⁸ Moreover, silencing different branches of flavonoid pathway in *M. truncatula* showed that the flavonol such as kaempferol is most likely to inhibit auxin transport during nodulation.⁶⁹ All these evidences support that flavonoids play a critical role in regulating cell divisions during nodule development in legumes.⁷⁰

Flavonoids and Actinorhizal Symbiosis

Actinorhizal plants are dicotyledons distributed among four angiosperm orders (Fagales, Cucurbitales, Fabales and Rosales), 8 families and 24 genera.⁷¹ Actinorhizal plants are woody species, with the exception of *Datisca* spp, which are herbaceous perennials. They play important roles in land reclamation, forestry, agroforestry and horticulture. These plants are capable of

forming root nodules as a result of infection by a nitrogen fixing actinomycete called *Frankia*.⁷² Statue of research on actinorhizal species was recently reviewed by Pawlowski et al.⁷³

Role in signaling? In actinorhizal plants, although the involvement of flavonoids in symbiosis is poorly understood, some evidence of a role in chemo-attraction and proliferation of *Frankia* has been reported in reference 24. Benoît and Berry⁷⁴ showed that flavonoid-containing preparations from seed washes of red alder (*Alnus rubra*) enhanced nodulation by *Frankia* in this species. These results were reinforced by the observation that flavonols (quercetin and kaempferol) contained in black alder (*Alnus glutinosa*) root exudates are able to enhance the level of nodulation.⁷⁵ Moreover, it was demonstrated that root hair curling is enhanced by exposure of *Frankia* to *A. glutinosa* root filtrate.^{76,77} Recently, the strain specificity in the *Myricaceae-Frankia* symbiosis was found to be correlated with plant root phenolics.⁷⁸ The main plant compounds differentially affected by *Frankia* inoculation are phenols, flavonoids and hydroxycinnamic acids. This work provides evidence that during the initial phases of symbiotic interactions, *Myricaceae* plants adapt their secondary metabolism in accordance with the compatibility status of *Frankia* bacterial strains suggesting thus that flavonoids might determine the specificity of the microsymbionts.

Role in nodule functioning? The involvement of polyphenols in the *Casuarina glauca-Frankia* symbiosis was investigated.⁷⁹ Histological analysis revealed a cell specific accumulation of phenolics in *C. glauca* nodule lobes, creating a compartmentation in the nodule cortex. Histochemical and biochemical analyses indicated that these phenolic compounds belong to the flavan class of flavonoids. It was shown that the same compounds were synthesized in nodules and uninfected roots. However, the amount of each flavan was dramatically increased in nodules compared with uninfected roots. The use of in situ hybridization established that chalcone synthase transcripts accumulate in flavan containing cells at the apex of the nodule lobe. Laplaze et al.⁷⁹ hypothesized that cell specific flavan biosynthesis and accumulation delimit cortical compartments containing *Frankia*-infected cells and might restrict endophyte invasion. The meaning of this compartmentation is not understood, but obviously some signal exchange with the endophyte is needed for its development. In parallel, it was found that the gene coding for chalcone isomerase (CHI) in *Elaeagnus umbellata* was highly expressed in root nodules, with levels increasing during nodule development.⁸⁰ The higher expression level of this gene in root nodules was proposed to be associated with defense mechanism against infection by *Frankia* or signal molecules in actinorhizal symbiosis.

More recently, the analysis of a *C. glauca* root and nodule expressed sequence tag (EST) database led to the identification of 8 genes coding for enzymes involved in the flavonoid biosynthesis pathway: chalcone synthase (*CgCHS1*), chalcone isomerase (*CgCHI*), isoflavone reductase (*CgIFR*), flavonone-3-hydroxylase (*CgF3H*), flavonoid-3'-hydroxylase (*CgF3'H*), flavonoid-3',5'-hydroxylase (*CgF3'5'H*), dihydroflavonol-4-reductase (*CgDFR*) and flavonol synthase (*CgFLS*). A kinetic study of the expression of these genes during *C. glauca* root inoculation with *Frankia* linked with a biochemical study of the

flavonoid composition of inoculated roots is consistent with the implication of flavonoids during actinorhizal symbiosis.^{81,82}

Conclusion

From the results above described, it is evident that even if the role of flavonoids has been well characterized in Rhizobium/legume symbiosis, several question marks still remain for their role in the AM and actinorhizal symbioses (Fig. 2). Since strigolactones have been identified as signaling compounds at the pre-symbiotic stage the role of flavonoids present in the root exudates could be to regulate later stages of the AM interactions.⁴⁷ In actinorhizal symbioses with activation of several genes of the flavonoid biosynthetic pathway in early stages of the interactions in concert with accumulation of flavan in root nodules argue for a multiple role as in legumes. Inhibition of the flavonoid pathway using RNA interference has been successful for deciphering the crucial roles of flavonoids during legume nodulation,⁷⁰ the same approach could be used regarding AM and actinorhizal symbiosis. From the plant side, in view of the recent finding of a common symbiotic signaling pathway for actinorhizal, legume and arbuscular mycorrhization,^{83,84} a major challenge is to determine the symbiotic functions of flavonoids both common and specific to the three root endosymbioses.⁸⁵ This could help to define strategies to optimize root-rhizosphere interactions or to transfer the ability to fix nitrogen in cereal crops in manipulating the flavonoid pathway.^{85,86}

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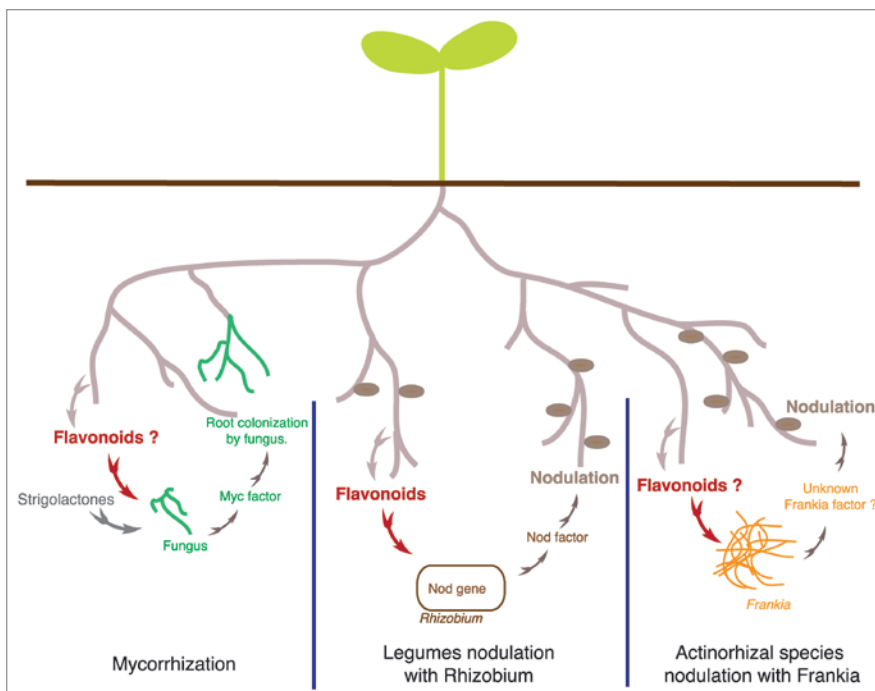


Figure 2. Schematic overview of flavonoid functions in the establishment of plant root endosymbioses. Flavonoids play an essential role in rhizobium-legume symbiosis as chemoattractant and *nod* gene inducers. They are suggested to act on mycorrhization by stimulating germination of spores and hyphal growth. The recent identification of strigolactones as host-recognition signals for AM fungi raises the questions about flavonoids as signal molecules in AM endosymbioses and of a possible interaction with strigolactones. Recent data suggest also a role for flavonoids in actinorhizal symbioses.

Acknowledgements

Financial support was provided by IRD and the Agence Nationale de la Recherche (Project SESAM 2010 BLAN 1708 01). K. Abdel-Lateif was supported by a fellowship from the Egyptian government.

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